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Brief Communication

Ability of Oligonucleotides with Certain Palindromes to Induce Interferon Production and Augment Natural Killer Cell Activity Is Associated with Their Base Length

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ABSTRACT

A synthetic 30-mer single-stranded oligodeoxyribonucleotide with a hexamer palindrome, AACGTT, induced IFN production and augmented NK activity in murine splenocytes. This effect does not appear to result from an antisense mechanism but rather is due to the palindrome. To clarify the required minimal size of the nucleotide, 10 kinds of 12- to 30-mer nucleotides were examined. Immunostimulatory activity of oligonucleotides 18 bases or more in length was observed and was proportional to the base length, with a maximum at 22–30 bases. On the other hand, the oligonucleotides 16 bases or less in length were not active even if they possessed the palindromic sequence. These results indicate that the immunostimulatory activity of oligonucleotides with certain palindromic sequences requires an oligonucleotide at least 18 bases long.

INTRODUCTION

A DNA-RICH FRACTION extracted and purified from *Mycobacterium bovis* BCG, MY-1, showed strong antitumor activity against various syngeneic tumors in mouse and guinea pig (Tokunaga et al., 1984; Shimada et al., 1985). This fraction augmented *in vitro* natural killer (NK) cell activity of murine spleen cells and human peripheral blood (Shimada et al., 1986; Mashiba et al., 1988; Yamamoto et al., 1988). It also induced production of interferon α (IFN-α) with small amounts of IFN-β and -γ and macrophage-activating factor (Mashiba et al., 1988; Yamamoto et al., 1988). These activities were destroyed by treatment of MY-1 with DNase, but not with RNase (Yamamoto et al., 1988). Thus the DNA component of MY-1 was considered to be responsible for the biological activities.

Previously we synthesized a variety of single-stranded oligodeoxyribonucleotides (oligoDNAs), sequences of which were chosen from cDNA encoding the 64-kDa heat shock protein (Ag A) of BCG (Thole et al., 1987), to assess the biological activity (Tokunaga et al., 1992; Yamamoto et al., 1992). One 45-mer oligoDNA (nucleotides 813–857 of the cDNA of Ag A), designated BCG-A4, augmented NK activity of murine spleen cells *in vitro*, whereas another 45-mer oligoDNA (nucleotides 694–738 of the same cDNA), designated BCG-A2, did not. Moreover BCG-A4a, which was a 30-mer oligoDNA with the 5′-end sequence of BCG-A4 and which possessed one palindromic sequence (GACGTC), was also active whereas BCG-A4b, which was a 30-mer with the 3′-end sequence of BCG-A4 and which possessed no palindromic sequence, was inactive. Fifteen-mer nucleotide fragments of BCG-A4, even if some of them possessed the palindromic sequence, had no activity (Yamamoto et al., 1992). These findings suggested that a unique palindromic sequence and some molecular size of synthetic oligoDNA was required to induce the biological activity. Furthermore, the hexamer palindromic sequence GACGTC in BCG-A4a (the sequence of which is 5′ ACCGATGACGTCGCCGGTGACGGCACCACG 3′) was replaced with 64 different palindromic base combinations. Of the 64 palindromes, only 9 palindromic sequences supported the biological activity of the 30-mer DNAs (Kuramoto et al., 1992). This finding suggested that the 9 potent oligoDNAs did not act by antisense mechanisms, and it also indicated that a variety of steric

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structures among the 64 kinds of hexamer palindromes, or some unique structure(s) of oligoDNA(s), might be responsible for the biological activity. On the basis of these results, we selected two BCG-A4a analogs designated AAC-30 and ACC-30 (Table 1) for further study of the steric structures and biological activity of oligoDNAs. AAC-30, having the AACGTT palindrome, is different by only two bases from ACC-30, having the ACCGGT palindrome; but the former induced IFN production and NK cell augmentation whereas the latter did not (Kuramoto et al., 1992; Yamamoto et al., 1992). In this study, we synthesized 13 kinds of oligoDNAs of various base lengths and determined the minimal molecular size of oligoDNA required for induction of IFN production and augmentation of NK activity.

Oligodeoxyribonucleotides were synthesized with an automatic synthesizer (Gene Assembler Plus; Pharmacia-LKB, Uppsala, Sweden) by the standard phosphoramidite method (Agrawal et al., 1989). After purification by gel filtration, dialysis, and ethanol precipitation, the oligoDNAs were lyophilized. Their concentration was determined by absorbance at 260 nm, after being resolved with saline. Purity of the oligoDNAs was confirmed by high-performance liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE). MY-1 was prepared as described previously (Tokunaga et al., 1984). Normal BALB/c female mice (8–12 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). Mouse lymphoma YAC-1 cells and fibroblast L929 cells were maintained in tissue culture using RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Assay for IFN level and NK activity was done as described previously (Yamamoto et al., 1992). Briefly, mouse spleen cells at a concentration of 1×10^7 cells/ml were incubated with 5×10^{-9} M (or 50 μg/ml) oligoDNA for 20 hr at 37°C. The cells were used as NK cells and the levels of IFN in the culture supernatants were measured. Natural killer cell activity was calculated as percent lysis, using ^{51}Cr-labeled YAC-1 cells as target. One million cultured spleen cells were mixed with 1×10^4 target cells in a final volume of 0.2 ml and distributed to the wells of a 96-well U-bottomed plate, and the cell mixture were cultured for 4 hr at 37°C. After incubation, the supernatants were collected and the radioactivity released from the target cells was measured by a standard γ scintillation counter. The percentage of target cell lysis was calculated by the following formula: [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100. The levels of IFN in the culture supernatants were measured in terms of the ability to inhibit cytopathic effects of vesicular stomatitis virus on mouse L929 fibroblasts. L929 cells (5×10^4) were seeded in a 96-well flat-bottomed plate in a volume of 50 μl. Twenty-four hours later, when the cells had become confluent, the wells were filled with additional 50-μl aliquots of twofold serial dilutions of the culture supernatants or the standard IFN, the titer of which was equivalent to the National Institutes of Health (NIH) reference IFN. After incubation for further 24 hr, the cultured L929 cells were treated with the virus in a 100-μl dilution containing 10 $TCID_{50}$ (50% tissue culture infectious dose). The cytopathic effect was examined microscopically after 40 hr of incubation. Antiviral units were expressed in international units (IU) per milliliter, which was calculated from the highest dilution for 50% inhibition of the cytopathic effect. Oligonucleotides had no direct effect on L929 cells. For preparation of AAC oligoDNAs of various base lengths, the extrapalindromic sequence of AAC-30 was trimmed stepwise. Trimming two nucleotides from the 3′ end of the AAC-30 sequence resulted in AAC-28. By repeating this procedure, AAC-26, AAC-24, AAC-22, AAC-20, AAC-18, AAC-16, AAC-14, and AAC-12 were selected. In these procedures, only extrapalindromic sequences were trimmed and a hexamer palindrome of AACGTT was maintained. Ten kinds of oligoDNA analogs having a hexamer palindromic sequence AACGTT were synthesized. Similarly, trimming of ACC-30 resulted in ACC-22 and ACC-20 (Table 1).

The activities of these oligoDNAs in inducing IFN production and augmenting NK cell activity are shown in Table 2. When murine spleen cells were cocultured with 5×10^{-9} M (or 50 μg/ml) ACC-30 analogs, seven oligoDNAs (AAC-30, AAC-28, AAC-26, AAC-24, AAC-22, AAC-20, and AAC-18) showed activity. On the other hand, AAC-14 induced slight NK activity but not IFN production, and AAC-12 showed no activity. In the case of AAC-16, the concentration was critical; 50

TABLE 1. SEQUENCE OF AAC AND ACC oligoDNAs OF VARIOUS BASE LENGTHS

Name	*Base sequence (5′ → 3′)*[a]				
AAC-30	ACCGAT	AACGTT	GCCGGT	GACGGC	ACCACG
AAC-29	ACCGAT	AACGTT	GCCGGT	GACGGC	ACCA
AAC-26	ACCGAT	AACGTT	GCCGGT	GACGGC	AC
AAC-24	ACCGAT	AACGTT	GCCGGT	GACGGC	
AAC-22	ACCGAT	AACGTT	GCCGGT	GACG	
AAC-20	ACCGAT	AACGTT	GCCGGT	GA	
AAC-18	ACCGAT	AACGTT	GCCGGT		
AAC-16	ACCGAT	AACGTT	GCCG		
AAC-14	ACCGAT	AACGTT	GC		
AAC-12	ACCGAT	AACGTT			
ACC-30	ACCGAT	ACCGGT	GCCGGT	GACGGC	ACCACG
ACC-22	ACCGAT	ACCGGT	GCCGGT	GACG	
ACC-20	ACCGAT	ACCGGT	GCCGGT	GA	

[a]Underlined sequences are palindromic.

TABLE 2. PRODUCTION OF INTERFERON AND AUGMENTATION OF NATURAL KILLER CELL ACTIVITY BY MURINE SPLEEN CELLS STIMULATED WITH AAC AND ACC oligoDNAs

	IFN (IU/ml)		NK activity[b] (% lysis ± SD)
OligoDNA[a]	5×10^{-9} M	50 μg/ml	(50 μg/ml)
AAC-30	128	128	25.8 ± 1.2
AAC-28	128	128	28.2 ± 1.0
AAC-26	128	128	29.6 ± 1.8
AAC-24	128	128	29.8 ± 1.7
AAC-22	128	128	30.0 ± 0.8
AAC-20	64	64	26.4 ± 0.5
AAC-18	8	32	18.3 ± 0.6
AAC-16	<4	4	13.9 ± 0.2
AAC-14	<4	<4	13.0 ± 0.5
AAC-12	<4	<4	11.6 ± 0.4
AAC-30	<4	<4	11.7 ± 1.3
AAC-22	<4	<4	11.4 ± 0.8
AAC-20	<4	<4	11.0 ± 0.6
MY-1	128	128	25.5 ± 1.0
Medium	<4	<4	10.4 ± 0.4

[a]The sequences of the oligoDNAs used are listed in Table 1.
[b]Spontaneous counts per minute was 1139 and total counts per minute was 9561.

μg/ml induced IFN production but a 5×10^{-9} M concentration, which is equal to 27 μg/ml, did not. The difference in concentration between 50 μg/ml and 5×10^{-9} M AAC-18 might have caused the difference in IFN level. None of the oligoDNAs having another palindromic sequence of ACCGGT (ACC-30, ACC-22, and ACC-20) showed the activity. These results suggested that the level of IFN was influenced by the concentration of intracellular potent oligoDNA, and it was confirmed by our experimental results of lipofection of AAC-22. When AAC-22 was encapsulated with liposome and transfected into murine spleen cells, the same level of IFN induced by 50 μg of naked AAC-22 per milliliter was induced by a low concentration of 0.016 μg/ml; lipofection was 3000-fold more efficient than coculture. Spleen cells transfected with liposome-encapsulated ACC-22 showed only a slight enhancement of IFN induction. Furthermore, when binding of AAC-22 to spleen cells was compared with that of ACC-22, no difference was observed between them (Yamamoto et al., 1993). These results strongly suggest that IFN production is triggered by entry of AAC-22 inside spleen cells but not by binding to cell surface receptors.

The NK activity augmented by AAC oligoDNAs was elevated with the level of IFN. But the NK activity augmented by AAC-30 was weaker than that of AAC-28, although the level of IFN was same. And in AAC-14 a slight augmentation of NK activity was recognized without IFN. Taken together with the indication from our previous study that IFN-α/β is responsible for the augmentation of NK activity by AAC-30 (previously designated A4a-AAC) (Yamamoto et al., 1992), the results shown in Table 2 suggest that another cytokine induced by AAC oligoDNAs may play a minor role in the augmentation of NK activity.

Eighteen- to 30-mer AAC oligoDNAs induced IFN in murine splenocytes *in vitro*, whereas AAC-12 and AAC-14 did not, even though they had the same palindromic sequence. These results indicate that the minimal base length of immunostimulatory oligoDNAs having a potent palindromic sequence (AACGTT) is 18, of which the molecular weight is about 6000. This molecular size may be required to maintain some unique steric structure to express the activity, and the structure may be more stable in AAC-20. The stability of the structure may reach an equilibrium state in AAC-22. BCG-A4a has a hexamer palindromic sequence (GACGTC), and this sequence is recognized by the restriction enzyme *PstI*. A hairpin structure of CT-GCAG in the synthesized 12-mer double-stranded DNA (dsDNA), having the sequence 5′ ACCCTGCAGGGT 3′, was observed by nuclear magnetic resonance (NMR) spectroscopy during the dissociation process from the double strand to single strand (H. Nakanishi [National Institute of Bioscience and Technology, Tsukuba, Japan], personal communication, 1993). This information supports our speculation that the potent palindromes may have some unique steric structure(s) to express the activity.

Although the reason why the difference in immunostimulatory activity between AAC oligoDNAs and ACC oligoDNAs was seen is still obscure, the studies on the steric structures of these oligoDNAs and expected regulatory protein(s) that bind AAC oligoDNAs will resolve the question. Further study of the steric structures of AAC-22 and ACC-22 by NMR spectroscopy is in progress.

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